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THE PRIMING EFFECTS OF TUMOR NECROSIS FACTOR AND INTERLEUKIN-1 ON CANINE NEUTROPHILS STIMULATED WITH INTERLEUKIN-8

Timothy Andersen

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ABSTRACT

Interleukin-8 (IL-8) is a novel neutrophil activating peptide that was recently identified in secretions from mononuclear phagocytes and endothelial cells. The purpose of this study was to determine the interplay between two well documented inflammatory mediators, Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1), and IL-8 in the priming of neutrophils for increased functional activity.

Neutrophil functional activity was measured using three laboratory assays. A vitamin B12 binding protein assay was used to detect content and/or degranulation of secondary granules, a chemotaxis assay was used for migration studies, and a dichlorofluorescein (DCF) hydrogen peroxide production assay was used to assess the respiratory burst function of the cell.

The priming results of TNF and IL-1 were compared to single cytokine stimulation. Means, standard errors of the mean, and tests for significance were used to compare data.

Results indicated that single cytokine stimulation with IL-8 and TNF produced increased functional activity compared to nonstimulated controls in all three assays. IL-1 stimulated increased degranulation.

Priming with IL-1 and TNF followed by IL-8 stimulation produced only limited synergistic effects when compared to

IL-8 stimulation alone. Cells primed with TNF at doses of 0.1 ng and 1.0 ng induced a significant increase in migration function when compared to direct TNF cytokine stimulation or a 37°C primed, IL-8 stimulated control. Priming cells with IL-1 did not produce increased migration nor degranulation responses. TNF primed cells did not demonstrate increased degranulation or hydrogen peroxide production.

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CHAPTER I.

INTRODUCTION

Cytokines

Cytokines are a family of heterogeneous glycoproteins that stimulate many cell types. Cytokines serve to stimulate the production, maturation, and recruitment of hematologic cells. These growth factors are usually several hundred amino acids long and most are glycosylated. T-lymphocytes, monocytes, and macrophages are the major producers of cytokines (1). They can also be produced to a lesser extent by a wide range of nonhematologic cells such as endothelial cells, renal cells, hepatic cells, and keratinocytes (2,3).

Most cytokines are grouped into two basic nomenclature schemes. Those factors in one class are most recently termed interleukin for their white blood cell to white blood cell signaling interaction. To date, Interleukins 1 through 12 have been characterized. The other class of cytokines uses the term "colony stimulating factor" preceded by the target cell upon which an effect is elicited. The major colony stimulating factors are "granulocyte macrophage-colony stimulating factor" (GM-CSF), "granulocyte-colony stimulating factor" (G-CSF), and "monocyte-colony stimulating factor" (M-CSF).

Cytokines signal other cells by receptor mediated transduction. For example, Interleukin-1 is a primary mediator of inflammation that signals the T lymphocyte or monocyte to produce Interleukin-2, Interleukin-8, and/or other cytokines (2). Cytokines act in either paracrine or autocrine fashion. These factors proceed to activate other cells which can produce even more cytokines. The stimulatory effect is a cascade of signals expanding in quantity and type, capable of pleiotropic regulatory ability. Cell response is both rapid and transient.

The cytokine transduction may also be detrimental. Excessive numbers of neutrophils accumulating in the area can lead to damage of the surrounding tissue. The same superoxide and hydroxyl radicals meant to digest invading bacteria can destroy surrounding healthy tissues. Unexpectedly high levels of Interleukin-8 have been demonstrated in lesions of psoriasis, in the pleural fluid of adult respiratory distress syndrome, and in synovial fluid of rheumatoid arthritis (4). Blocking agents such as glucocorticoids and antibodies directed at the cytokine usually lessen the overt symptoms. It remains unclear what triggers overproduction of Interleukin-8 and whether there may be an unknown benefit.

Certain cytokines possess the ability to induce increased production and functional activity of white

blood cells. Two such cytokines are granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Each has been shown to increase the production of polymorphonuclear neutrophils (PMNs) ten-fold following several days of administration of cytokine (5). The production of PMNs continues with administration of either cytokine, but returns to within normal levels several days after cessation of G-CSF or GM-CSF. A second function of these cytokines is their ability to upregulate or prime neutrophil metabolism to enhance microcidal ability. Studies on GM-CSF have reported increased superoxide production, phagocytosis, and lysozyme secretion (6).

TNF and IL-1 are examples of pro-inflammatory cytokines that can act indirectly by stimulating cells to release other cytokines such as G-CSF, GM-CSF, and IL-8. TNF and IL-1 also serve to prime for increased function of PMNs (7,8).

The full effects of Interleukin-8, individually or in synchrony with other cytokines, is yet to be discovered. Preliminary studies indicate that IL-8 directly stimulates PMNs for chemotaxis or the ability to migrate toward a chemoattractant, while data on degranulation and production of microbiocidal agents are still equivocal.

The Role of the Neutrophil in Inflammation

The polymorphonuclear neutrophil (PMN) is the major cell responsible for localizing, digesting, and killing invading microorganisms. It is also the prevalent leukocyte in the peripheral circulation. The sequence of processes that the neutrophil undergoes to eradicate invading microorganisms is a continuum, rather than a series of discrete events. When neutrophil receptors bind specific cytokines, the neutrophil undergoes a sequence of complex physiological changes, which can result in increased metabolic functions.

One of the first detectable events of cytokine stimulation is neutrophil adherence or margination. The neutrophil migrates from the free-floating circulation and attaches to endothelial cells near the site of inflammation. Cytokines such as Interleukin-1 (quite possibly the same one acting upon the neutrophil) are also capable of upregulating endothelial cell receptors to make them more "sticky" to the neutrophil (9). The neutrophil actively moves toward the vascular opening nearest the site of inflammation by means of projecting pseudopodia on the leading edge. Neutrophils can move in directed fashion in response to a cytokine concentration gradient increase as little as one percent from front to back of the cell (10).

The neutrophil can also respond to other chemoattractants such as anaphylatoxins and endogenous pyrogens (10). The neutrophil rapidly synthesizes new cytokine receptors and recycles old ones to enhance further detection of cytokine. Cytokine receptors are stored in the secondary granules which are maintained near the leading edge of the migrating cell (11). This pool of additional receptors can be fused to the plasma membrane as the cell migrates through a cytokine gradient. The neutrophil then crawls through the small vascular opening and continues to migrate to the site of inflammation.

Either during migration or once in the inflammatory area, cells are stimulated to produce enzymes that will be utilized in the respiratory burst phase to produce hydrogen peroxide, which kills and hydrolyzes microorganisms. The enzymes are stored in the secondary granules of the neutrophil until needed (10). Cell activation may result in degranulation of secondary or specific granules. The enzymes required to convert the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2) are fused and released into the phagosome. The granules may eventually be released extracellularly.

Literature Review of IL-8, TNF, and IL-1

Interleukin-8 is a newly described cytokine that participates in the inflammatory response (12). In concert with other cytokines, Interleukin-8 (IL-8) modulates the function of polymorphonuclear cells (PMNs) in the body's defense against invading microorganisms.

Interleukin-8 was described in the literature in 1987 by Yoshimura (13). Two different sources have been characterized, one form derived from endothelial cells and the other from monocytes. Several variant forms with considerable amino acid sequence homology have been described, most notably Neutrophil Activating Peptide-1 (NAP-1) and NAP-2 (14). Leonard (14) suggests that NAP-2 shares NAP-1 receptors, but has differing chemotactic abilities.

Interleukin-8 has been cloned and made available by recombinant technology using a synthetic gene expressed in *Escherichia coli* (15). IL-8 has been called by various names and only recently has there been an attempt to unite information under the single name Interleukin-8. Interleukin-8 has alternately been called "monocyte derived neutrophil activating peptide" (MDNAP), "neutrophil activating peptide" (NAP), "leukocyte adhesion inhibitor" (LAI), and "T lymphocyte chemotactic factor" (TCF). Sica (4) indicated that the monocyte derived form of IL-8 is

produced in response to Interleukin-1, tumor necrosis factor (TNF), and lipopolysaccharide (LPS). Gimbrone (16) described the same stimulants as capable of producing the endothelial form of IL-8. Other cells such as hepatocytes (3), T lymphocytes (18), dermal fibroblasts (2), and keratinocytes (9) may also yield IL-8.

Interleukin-8 elicits varying responses by several cells. At present most of the research centers on the effect on neutrophils. Larsen (17) demonstrated that IL-8 induces chemotactic responses by T lymphocytes, and to a lesser extent basophils.

Endothelial-derived neutrophils have approximately 7,000 receptors per cell specific for IL-8 (3). These receptors are not shared with other cytokines and have a disassociation constant (K_d) of 1.2×10^{-9} (14). Samanta (18) showed that the neutrophil has approximately 20,000 receptors for the monocyte derived form of IL-8 with a single type of high affinity binding (K_d of 8×10^{-10}M). In another article, Samanta (19) showed that monocyte derived IL-8 rapidly down-regulates the number of neutrophil receptors and that rapid recycling is essential for the chemotactic response of neutrophils. When bound, IL-8 induces the cell to marginate and to bind to endothelial

derived IL-8 inhibits this adhesion and serves to protect the endothelial lining from further damage.

Interleukin-8 is not species specific (9). It exhibits some functional effectiveness in several laboratory animals tested.

Extensive work has been conducted on the effects of tumor necrosis factor (TNF) and Interleukin-1 (IL-1). The ability of TNF to kill tumor cells was first utilized by Coley in 1891 (20). He noted that cancer patients who suffered certain bacterial infections experienced spontaneous tumor regression without damage to healthy tissue. Dr. Coley began to treat cancer patients with various mixtures of bacteria called "Coley's Cancer Cocktails". In 1936 Shear (21) isolated the bacterial endotoxin that elicited TNF production from the macrophage. Fong (7) more recently described the malaria killing ability of TNF. Endothelial cell derived TNF was successfully cloned in 1985.

Interleukin-1 is much more pleiotropic than TNF in its effects. IL-1 is produced by macrophages in response to antigen (1). IL-1 induces T lymphocytes to generate IL-2 that in turn stimulates T-cell proliferation. Interleukin-1 also acts upon early hematopoietic progenitors to make them more responsive to other cytokines, such as G-CSF and GM-CSF (1). There are two forms of IL-1; IL-1 alpha and

them more responsive to other cytokines, such as G-CSF and GM-CSF (1). There are two forms of IL-1; IL-1 alpha and IL-1 beta. Although they exhibit little homology, they appear to act upon the same receptor and have similar activities (1).

Sample (8) showed that using TNF and IL-1a as direct stimuli prompted increased hydrogen production in the neutrophil. He also indicated they can cause degranulation of primary and secondary granules. Yoshimura (13) contradicted earlier studies by showing that purified recombinant TNF and IL-1a do not have chemotactic activity with *in vitro* neutrophils.

Statement of the Problem

The effect of multiple cytokines on the circulating neutrophil will be examined in the present study. The *in vivo* neutrophil is subject to multiple stimulations of varying degrees. The purpose of this project is to mimic the *in vivo* situation by exposing neutrophils *in vitro* to primary and secondary stimuli. This project will attempt to verify the hypothesis that two cytokines used in sequence can stimulate greater neutrophil functional capabilities than single cytokine stimulation alone. The direct and/or priming effect of TNF and IL-1a on the neutrophil's functional response to Interleukin-8 will be

observed. The parameters to be measured are important physiological events in the activation of the neutrophil. The three established laboratory assays to be utilized in this study are: (1) a Vitamin B12 Binding Protein Assay to measure the extent of granularity, (2) a Chemotaxis Assay to measure the ability of directed neutrophil migration, and (3) a Respiratory Burst Activity Assay to measure the cell's capacity to produce hydrogen peroxide. This hypothesis will be examined by determining the dose and time related response of isolated neutrophils to recombinant human Interleukin-8 (rhIL-8). Using the optimum concentration and stimulation time for IL-8, the cells will then be primed with IL-1 and TNF and assayed for functional levels. The results of the three assays will be compared to single cytokine stimulation and against the recognized nonreceptor mediated stimulant phorbol myristate acetate (PMA). PMA is a phorbol ester that has been previously shown to activate the neutrophil respiratory burst function (22). PMA is a structural analog to diacylglycerol. As such, PMA bypasses surface receptors and directly binds to intracellular protein kinase C. Activation by protein kinase C can lead to several responses, such as degranulation, aggregation, and/or stimulation of the respiratory burst activity (23). PMA maximally activates the membrane bound NADPH-oxidase

enzyme, via protein kinase C, which is chiefly responsible for the respiratory burst function (25).

CHAPTER II.

METHODS AND MATERIALS

Subject Animals

Four female beagles, (*Canis familiaris*), 2 ± 0.2 (S.E.M.) years old, mean weight 8.5 kg ± 0.5 (S.E.M.) were housed in individual stainless-steel cages in conventional holding rooms. Each animal was quarantined and screened for evidence of disease before being released for experimentation. The mean daily white cell counts were 9.78 /ml ± 0.31 x 10^3 (S.E.M.) and the mean per cent of granulocytes was 64.65 ± 0.96 (S.E.M.). The dogs were provided 10 air exchanges per hours of 100 per cent fresh air, maintained at 21±2° with a relative humidity of 50±10 per cent. They were maintained on a 12 hour light/dark full-spectrum lighting cycle with no twilight. The dogs were provided commercial dog food and tap water *ad libitum*.

Neutrophil Isolation

Whole blood, 2-3 ml, was drawn from each dog's lateral saphenous vein into heparinized (1U/ml of blood) syringes. The red cells were lysed with approximately 45 ml of 0.83% NH_4Cl for 10 minutes at room temperature. The cells were centrifuged into a pellet for 10 minutes at 400xg on a

Beckman TJ-6R Centrifuge. The red cell lysate was discarded. The pellet was washed in 20 ml of cold Hanks' Balanced Salt Solution, without Ca^{2}+ or Mg^{2}+ (Hanks-, #310-4175AJ Gibco Laboratories, Life Technologies, Inc. Grand Island, NJ) and centrifuged as above. The supernatant was decanted and the cells were resuspended to an approximate concentration of 5-10 x 10^{6}/ml in Dulbecco's phosphate-buffered saline solution (PBS-, #310-4200AJ Gibco Laboratories). Prepared cells were kept on ice and out of the direct sunlight until analysis to prevent cell activation.

Total and Differential Cell Counts

Total cell counts on the isolated samples were determined electronically using a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). White blood cell (WBC) differentials and morphologic examinations were performed on each sample. Differential count slides were obtained with a Cytospin 2 centrifuge. A 50,000 cell aliquot was added to 100 ul fetal calf serum (FCS) in a cytospin cuvette. The sample was centrifuged for 3 minutes at 1250 rpm. The slides were allowed to air dry prior to staining with Wright Giemsa on an Ames Hema-Tek automated slide stainer (Ames Industries, Elkhart, IN).

Vitamin B12 Binding Protein Assay

Degranulation, or conversely, cell granularity was assessed by the method described by Gottlieb *et al*. (24). The amount of vitamin B12 binding protein (B12) contained in the secondary granules was assessed utilizing a ^{57}Co-vitamin B12 binding assay. The amount of the gamma irradiation emitted by the ^{57}Co was therefore an indirect measure of secondary granules contained in the neutrophils.

Three separate microfuge tubes with 1 x 10^6 neutrophils per ml were prepared from each specimen. "Total", "stimulated", and "control" tubes were prepared using PBS as the diluent. The "total" tube represented the amount of B12 released when the neutrophil was lysed. The "stimulated" tube represented the amount of B12 released by the cell as a result of cytokine stimulation at the prescribed incubation. The "control" tube demonstrated the spontaneous release of B12 in response to incubation exclusively.

The stimulant (IL-1, IL-8, TNF, or PMA) was added to the "stimulated" tube. The "stimulated" and "control" tubes were incubated for 10 minutes in a 37^{o} waterbath. The tubes were then placed on ice for one to two minutes to stop the release function. The "stimulated" and "control" tubes were spun in a serofuge (Eppendorf Centrifuge 5415)

at the highest setting (14,000rpm) for one minute. Centrifugation removed the cells with any intracellular B12 binding protein and left only B12 in the supernatant. Duplicate 200 ul aliquots of supernatant were carefully pipetted into clean tubes. The "total" tube was mixed prior to pipetting to ensure an even suspension of cells was transferred to the clean tube.

The cells were lysed with one hundred ul of 0.2% Triton X-100 (#111036 Research Products International, Ltd., Elk Grove Village, IL) added to 200 ul aliquots of cell suspension. Lysing with triton released the maximum amount of B12. These tubes were labeled "total" tubes and duplicate 200 ul aliquots of the mixture were pipetted into clean tubes.

Hanks' Balanced Salt Solution (HBSS+) with Ca^{2+} and Mg^{2+} (Gibco Laboratories, Inc.) was added to bring all tube volumes to 1 ml. All tubes were mixed again.

^{57}Co-vitamin B12 (Cyanocobalamin Solution, Intermediate Specific Activity, CT-12, Amersham), 100 pg, was added to all tubes. The samples were again mixed and incubated at room temperature for 30 minutes. The ^{57}Co bound spontaneously to the released B12.

Charcoal suspension, 800 ul of a 2.5% solution in 0.5% albumin (Activated Carbon, Norit A, Aldrich Chemical

Company, Inc.), was added to the tubes and mixed. The tubes were incubated an additional ten minutes at room temperature to allow the charcoal to bind any unbound ^{57}Co-vitamin B12. The charcoal suspension does not to bind the vitamin B12-^{57}Co-binding protein complexes (22).

The samples were centrifuged (Beckman TJ-6R Centrifuge, Beckman Industries) at highest speed (approximately 4000rpm) for 25 minutes to remove the charcoal containing free ^{57}Co.

One ml aliquots of supernatant were pipetted to clean tubes and the tubes capped. The gamma irradiation emitted from ^{57}Co in the supernatant was counted on a gamma scintillation counter (1282 CompuGamma CS Sodium Iodide Detector (NaI), LKB Pharmacia) for one minute.

The radioactivity counts from duplicate tubes were averaged and the pg of vitamin B12 contained in each sample were calculated first by subtracting the CPM of a charcoal blank tube from the CPM of each assay tube. The net CPM was converted to pg using the specific activity of the ^{57}Co-vitamin B12. The per cent release of the vitamin B12 binding protein was assessed indirectly as:

$$\frac{\text{Stimulated value X 100}}{\text{Total value}}$$

Chemotaxis Assay

The method used to quantitate the number of neutrophils migrating in response to chemoattractant was adapted from a technique described by Harvath, Falk, and Leonard (25).

Granulocyte suspensions of 1 x 10^6 cells were prepared in one ml of either buffer (RPMI Medium 1640, Gibco Laboratories) or chemoattractant (IL-8, Zymosan activated plasma, TNF, or IL-1a). Heat inactivated, zymosan activated plasma (ZAP) is an established chemoattractant for canine neutrophils (23). Mature segmented neutrophils, band neutrophils, and eosinophils were included in the granulocyte calculation since each of these cell types have been shown capable of migration.

Buffer, 29.6 ul, was pipetted into the first three wells of a row in a specially designed 48-well Chemotaxis Chamber (Neuroprobe Inc. Cabin John, MD). The appropriate chemoattractant, 29.6 ul, was pipetted into the next six wells of the chamber (Figure 1).

A polycarbonate, polyvinylpyrrolidone-free membrane with 3 um pores (PC PVPF 25x80mm, Nucleopore Corp.) was then placed over the wells. A thin rubber gasket with holes cut the same size as the wells was laid over the top of the membrane. The top of the chamber was placed over

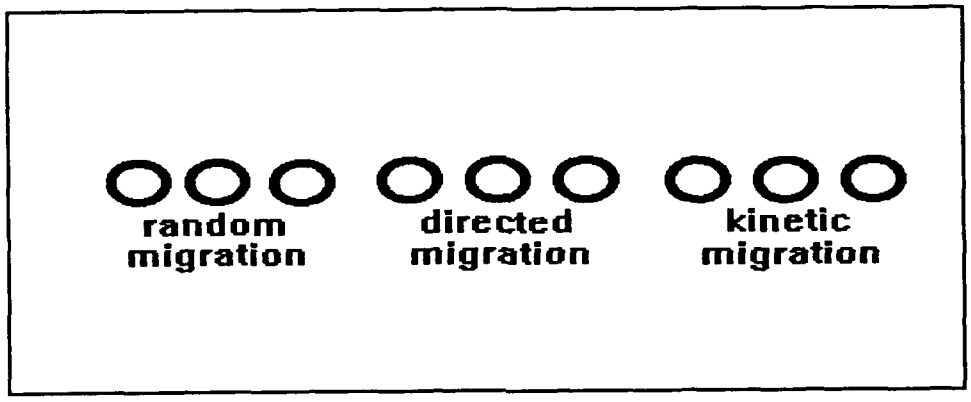


Figure 1. Row configuration of chemotaxis wells.

the gasket and affixed firmly with six wing-nut screws. The top of the chamber contained the uppermost half of the wells into which the cells were placed. Cells diluted in buffer, 50 ul, were pipetted into the first six wells of the row. Cells diluted in chemoattractant, 50 ul, were placed in the last three wells (Figure 2).

The chemoattractant/cell suspension required mixing just prior to pipetting into the wells to preclude additional priming by the chemoattractant.

The resultant arrangement of the triplicate well types was as follows: (1) The first three wells had cells diluted in buffer suspended above the membrane over buffer. These wells represented random migration, as the cells in these wells were not exposed to any chemoattractant. (2) The center three wells had cells diluted in buffer over chemoattractant. These wells represented directed migration. The cells that migrated in response to the cytokine gradient and crawled through the 3 um pores adhered to the underside of the membrane. (3) The last three wells had cells diluted in chemoattractant suspended over chemoattractant, and represented chemokinetic migration. The cells were activated by the cytokine, but did not have gradient-directed migration.

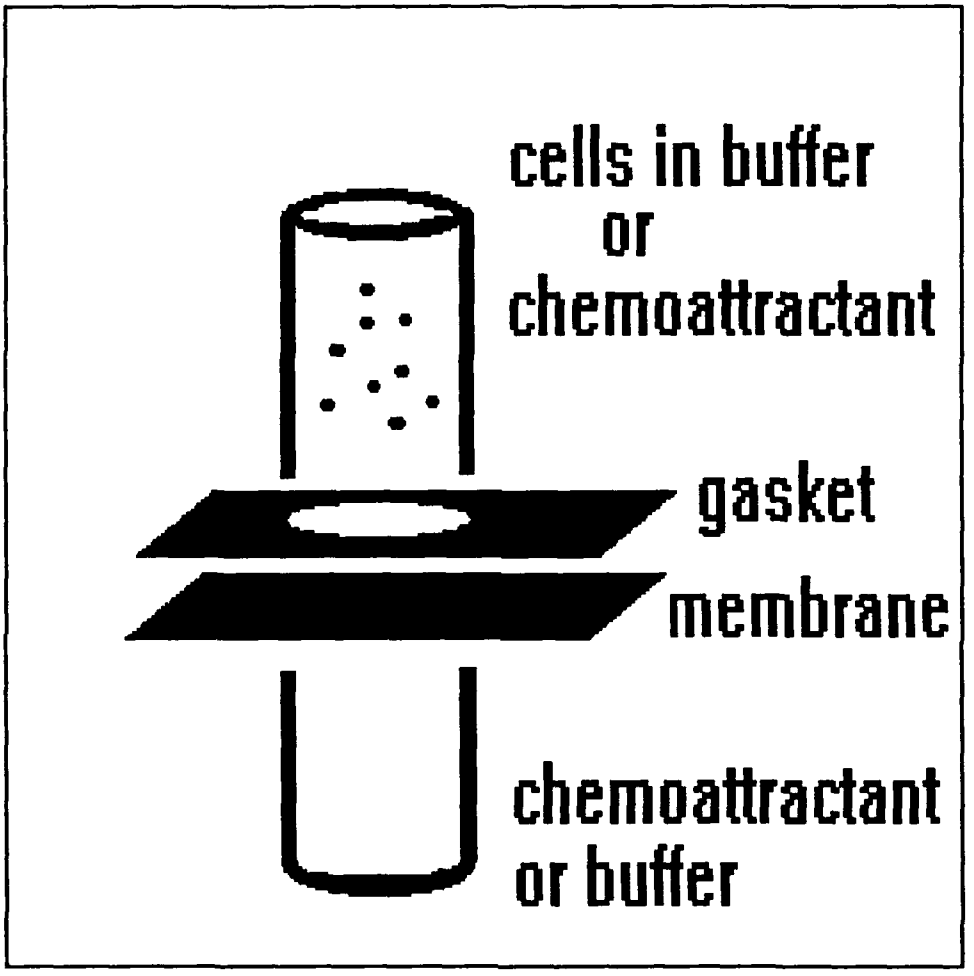


Figure 2. Well configuration of chemotaxis assay

The chamber was incubated for one hour in a 37°C incubator. The screws were then removed and the chamber inverted and disassembled. The migrated cells were now on the upper side of the membrane. The membrane was lifted and the nonmigrated side of the membrane was touched to Hanks- and then gently scraped across a rubber scraper. This procedure was repeated three times to remove the cells which had not migrated though the pores to the chemoattractant. The membrane was fixed for one minute in Diff Quik Fixative (Baxter Scientific Products) and then stained with Diff Quik Solutions I (1 g/L xanthene dye) and II (1.8 g/L triarylmethane dye in 100% methyl alcohol) (Fisher Scientific) for one minute in each solution. Excess stain was removed by rinsing the membrane in distilled water.

The membrane was inverted and placed on a 3x2 inch pre-cleaned Opticlear Microscope slide (Kimble). Any wrinkles were gently removed and the membrane was left to air dry completely. Immersion oil (Sp. Gr. 1250) was added to the top of the membrane and a coverslip applied. The slide was then placed on an Olympus microscope attached to an Optomax 40.10 Image Analyser (Optomax Inc., Hollis, NH). Four consecutive fields of each well were counted using the 40x objective (400x power total).

The Optomax automated the counting procedure by light tagging the cells in the field. The light tags which could be adjusted to a 1:1 (cell:light tag) ratio by means of a rheostat, were electronically counted by the Optomax.

Four consecutive field counts were taken for each of the three wells. The four counts were averaged and then the mean of the three wells was calculated. Each data point represented 12 counts.

H2O2 Production

The cell's respiratory burst activity was determined by measuring the amount of hydrogen peroxide (H_2O_2) produced after the cells were stimulated with cytokine. Hydrogen peroxide production was measured by the technique described by Bass *et al*. (26) using a flow cytometer (FACS Analyzer, Becton Dickinson, Mountain View, CA).

Cell suspensions containing one million cells were added to one ml total volume of PBS-, 5 mM glucose, and 5 uM dichlorofluorescin diacetate (DCFH-DA) dye. The uncharged, nonfluorescent, nonpolar dye, DCFH-DA was added to the cell suspensions where it passively diffused through the cell membrane and was converted to the nonfluorescent esterase 2',7'-dichlorofluorescin (DCFH). Once converted, the intermediate DCFH compound was not able to exit the cell. All

samples were mixed and incubated for ten minutes in a covered, 37°C waterbath to allow for dye equilibration. Following incubation, the samples were kept at 4°C, light protected, and assayed immediately.

The specimens were aspirated to the flow cytometer and 10,000 events were collected. A threshold was set for volume (linear scale) and a live gate set for fluorescence (log scale) to remove any debris from being mistaken for cells. The equilibrated DCF dye in the cells was excited by the mercury arc lamp on the flow cytometer. Green fluorescence was monitored with a 485/22 nm excitation filter. The samples were measured before (control) and after (experimental) stimulation.

Each specimen was measured at time = 0 (T_0). The stimulant (IL-1a, TNF, IL-8, or PMA) was then added and the samples were assayed at several time periods (T_{15}, T_{30}, etc.). Between measurements the specimens were incubated in a light protected, 37°C waterbath for the appropriate incubation time. The data on neutrophil events were separated from the data of other cells by means of gating. The acquired events were first subjectively gated based on established criteria of cell granularity and volume. The side scatter of light (RTO) is a measure of the cell's granularity. RTO gating ensures that neutrophils with

significant granularity were isolated from other cell types lacking granularity. Gating by volume separates cells by their size.

The data were analyzed by a Consort 30 computer interface (Becton Dickinson). The weighted mean peak of fluorescence of the gated neutrophils was calculated by the Consort 30. The change in H_2O_2 production was determined from the mean peaks of fluorescence taken before (T_0) and after (T_{15}) stimulation using the following formula:

$$\frac{\text{Stimulated } (T_{15}) - \text{Unstimulated } (T_0)}{\text{Unstimulated } (T_0)}$$

Statistical Analysis

The results were expressed as the mean $\pm$S.E.M. for the data obtained from separate experiments. The probability of significant differences between single cytokine stimulation values versus unstimulated control values and cytokine primed, stimulated values versus 37°C primed, stimulated control values were established using paired t-tests. Statistical significance was taken at the 5% level.

CHAPTER III.

RESULTS

Vitamin B12 Binding Protein Release

Direct Stimulation Studies

Variability of the Method

The coefficient of variation (C.V.) and standard error of the mean (S.E.M.) were calculated for each of the four animals from ten determinations taken on separate days. Each specimen was stimulated with 25 ng of PMA for ten minutes at 37°C (Table 1).

Incubation Time Response

The incubation time required to ensure the maximal release of Vitamin B12 binding proteins was determined by stimulating the PMNs of the four dogs with 25 ng of PMA and incubating the cells at ten and twenty minutes. Assays were conducted over several days (Table 2).

A paired t-test of the two columns did not indicate a significant difference ($p=.51$) between the two incubation times. Therefore, a ten minute incubation was used for all subsequent direct stimulation studies.

Dose Response Curves

Dose response curves were run using the ten minute

TABLE 1.

Periodic Measurements of B12 Per Cent Release from the Neutrophils of Four Canines Stimulated with 25 ng PMA

CHJ09		CDF97		CCH41		CGF76	
Date	%Release	Date	%Release	Date	%Release	Date	%Release
4-1	82	4-1	77	4-1	80	4-1	70
4-4	79	4-2	71	4-4	91	4-4	70
4-11	56	4-4	68	4-15	62	4-11	60
4-19	64	4-15	53	4-22	55	4-19	62
4-23	68	4-22	57	4-24	54	4-19	55
4-23	68	4-24	62	4-26	54	4-23	56
4-25	72	4-30	58	4-30	82	4-25	60
4-29	59	5-2	71	5-2	51	4-29	78
5-1	43	5-6	77	5-6	61	5-1	48
5-7	66	5-10	67	5-10	56	5-7	56
MEAN	65.7		64.2		64.4		61.5
S.E.M.	$\pm$3.6		$\pm$2.5		$\pm$4.5		$\pm$2.8
C.V.	17.2%		13.6%		22.1%		14.4%

TABLE 2.

The Effect of Incubation Time on B12 Per Cent Release on Neutrophils from Four Canines Stimulated with 25 ng PMA

Animal	Date	% Release at 10 min	% Release at 20 min
CHJ09	4-1	82	69
	4-4	79	71
CDF97	4-4	68	83
	4-15	77	68
CCH41	4-4	80	97
	4-15	62	68
CGF76	4-1	70	74
	4-4	70	75
MEAN±SEM		73.5±2.5	75.6±3.5

incubation at 37°C for the three cytokines (IL-8, IL-1, and TNF) and PMA (Figures 3-4 and Table 3). Each data point represents the mean of the values from four animals from at least two replicates assayed on different days.

The three cytokines each elicited an increased per cent release as compared to a 37°C control.

Tumor Necrosis Factor stimulated the greatest amount of release of vitamin B12 binding proteins at 100 ng. The response produced by TNF was higher at all three concentrations tested compared to IL-1 and IL-8. The maximal response for IL-1 was at 10 ng and for IL-8 at 100 ng. Therefore, 100 ng was the concentration used for IL-8 stimulation for all subsequent priming studies.

As is frequently reported in the literature (25,27), PMA stimulates a much greater response that any of the three cytokines.

Priming Studies

Response of Priming Time and Primer Concentration

The effect of priming time for 0.1, 1.0, and 10 ng of TNF (Figure 5) and IL-1 (Figure 6) was determined by priming each of the four specimens at 5, 30, and 60 minutes at 37°C (Table 4), followed by a ten minute stimulation

Figure 3. B12 degranulation produced by IL-1 and TNF used as direct stimulants.

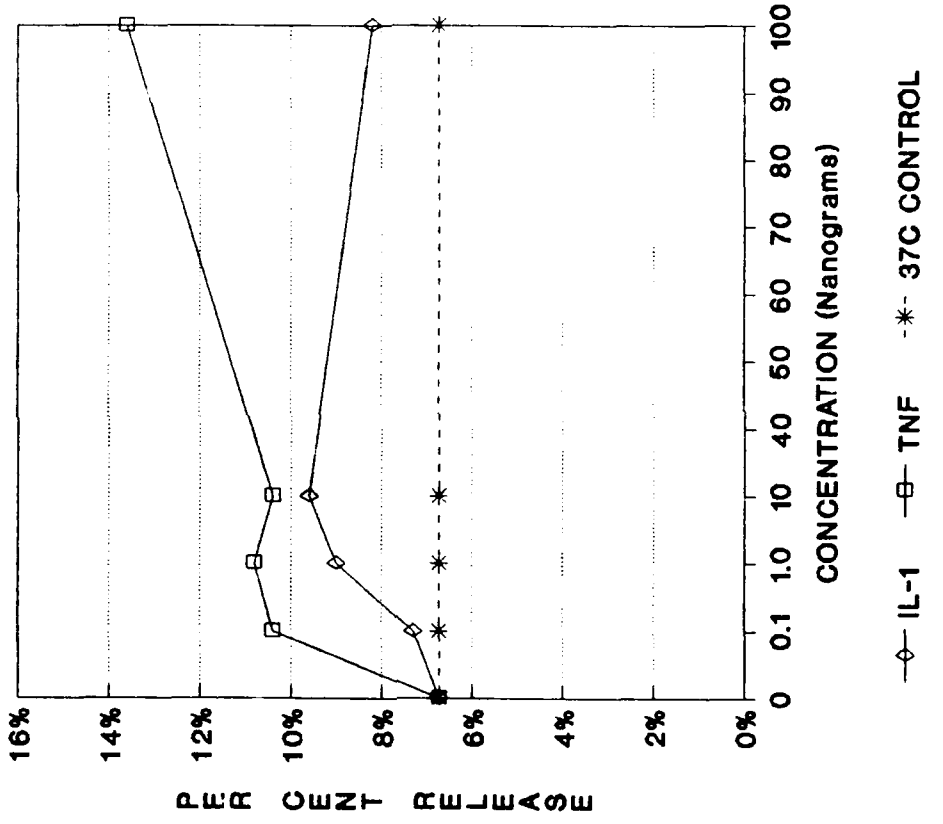

PER CENT RELEASE
16%
14%
12%
10%
8%
6%
4%
2%
0%
0
0.1
1.0
10
40
50
60
70
80
90
100
CONCENTRATION (Nanograms)
IL-1
TNF
37C CONTROL

Figure 4. B12 degranulation produced by IL-8 and PMA used as direct stimulants.

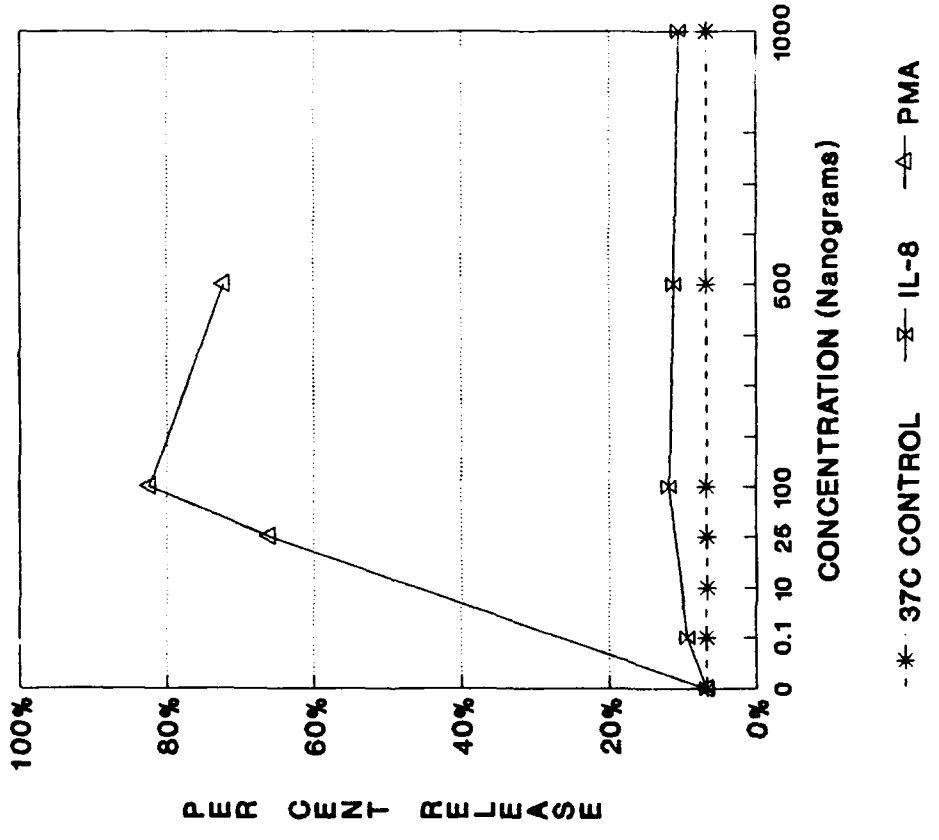

PER CENT RELEASE
100%
80%
60%
40%
20%
0%
0
0.1
10
25
100
600
1000
CONCENTRATION (Nanograms)
37C CONTROL
IL-8
PMA

TABLE 3.

B12 Per Cent Release of Various Concentrations of IL-8, TNF, IL-1, and PMA Used as Direct Stimulants

	0.1 ng	1.0 ng	10 ng	25 ng	100 ng	500 ng	1000 ng
IL-8	9.4 ±1.4	NT NT	NT NT	NT NT	11.8 ±1.0	11.2 ±1.4	10.5 ±1.2
TNF	10.4 ±1.1	10.8 ±0.9	10.4 ±1.1	NT NT	13.6 ±1.7	NT NT	NT NT
IL-1	7.3 ±0.8	9.0 ±0.9	9.6 ±1.1	NT NT	8.2 ±0.4	NT NT	NT NT
PMA	NT NT	NT NT	NT NT	66.1 ±2.4	82.2 ±2.0	72.2 ±4.6	NT NT
37C	6.7 ±0.6	6.7 ±0.6	6.7 ±0.6	6.7 ±0.6	6.7 ±0.6	6.7 ±0.6	6.7 ±0.6

NT= not tested

Figure 5. B12 degranulation produced by TNF priming and 100 ng IL-8 stimulation.

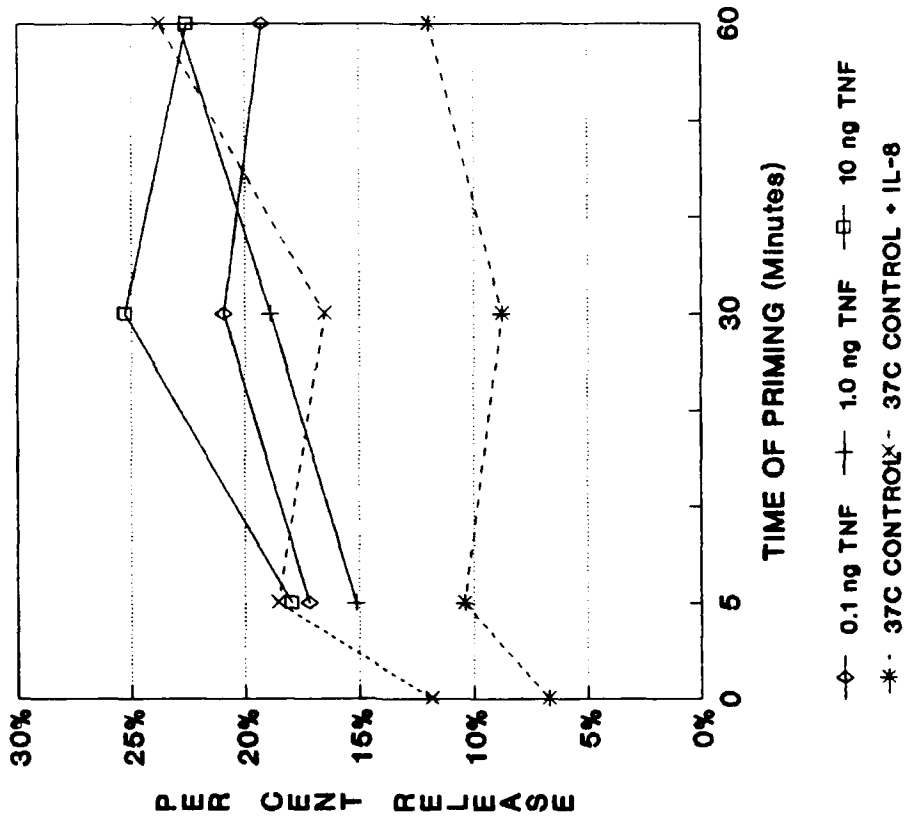

PER CENT RELEASE
30%
25%
20%
15%
10%
5%
0%
0
5
30
60
TIME OF PRIMING (Minutes)
0.1 ng TNF
1.0 ng TNF
10 ng TNF
37C CONTROL
37C CONTROL + IL-8

Figure 6. B12 degranulation produced by IL-1 priming and 100 ng IL-8 stimulation.

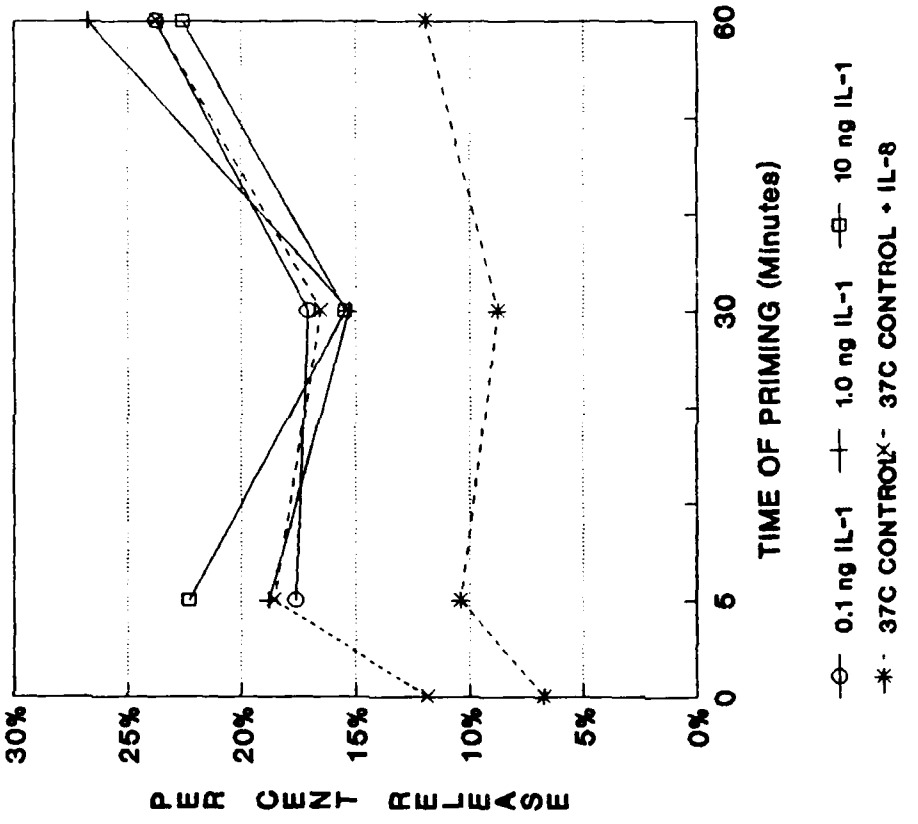

PER CENT RELEASE
30%
25%
20%
15%
10%
5%
0%
0
5
30
60
TIME OF PRIMING (Minutes)
0.1 ng IL-1
1.0 ng IL-1
10 ng IL-1
37C CONTROL
37C CONTROL + IL-8

TABLE 4.

B12 Per Cent Release Stimulated by 0.1, 1.0, and 10 ng Concentrations of TNF and IL-1 Priming for 5, 30, and 60 Minutes, Followed by 100 ng IL-8 Stimulation

Minutes	0	5	30	60
TNF				
0.1 ng	NT	17.2±2.5	20.9±3.5	19.2±1.5
1.0 ng	NT	15.1±2.1	18.9±2.6	22.8±2.7
10 ng	NT	18.0±2.2	25.9±3.2	22.5±2.1
IL1				
0.1 ng	NT	17.6±1.1	17.1±1.1	23.8±6.5
1.0 ng	NT	18.8±2.1	15.3±1.3	26.8±7.0
10 ng	NT	22.3±2.5	15.4±1.8	22.6±4.9
37C	11.8±1.0	18.6±1.7	16.5±1.7	23.7±2.9

NT = not tested

with 100 ng of IL-8.

TNF primed neutrophils, at all three concentrations, demonstrated the highest per cent of release when compared to IL-1. The maximum response was seen in cells primed with of TNF for 30 minutes. This data point also had the greatest divergence from its nonprimed, stimulated 37°C control. However, it was not statistically different when evaluated by a paired t-test ($p=0.069$).

The priming of neutrophils with IL-1 over a time course resulted in lesser amounts of release than TNF. The maximum response for B12 release occurred when cells were primed with IL-1 at a concentration of 1.0 ng for 60 minutes. There were no appreciable differences in response between the priming time periods.

Nearly all data points for IL-1 primed cells were appreciably greater than their nonprimed, nonstimulated, and time-matched 37°C controls (Table 5).

However, none of the concentrations for TNF or IL-1 were significantly elevated above the 37°C primed, time-matched controls stimulated with 100 ng of IL-8.

Chemotaxis

Variability of the Method

TABLE 5.

Statistical Significance P Values of B12 Per Cent Release Measurements of 0.1, 1.0, and 10 ng IL-1 Priming, Followed by 100 ng IL-8 Stimulation Compared to 37°C Primed, IL-8 Stimulated, Time-Matched Controls

Concentration	0.1 ng	1.0 ng	10 ng
5 min Priming	.007	.013	.012
30 min Priming	<.0001	.0007	.0064
60 min Priming	N.S.	.0125	.0106

N.S.= Not Significant

The coefficient of variation and standard error of the mean were calculated for each of the four animals using 100 ng of IL-8 as chemoattractant (Table 6).

Dose Response Curves

Dose response curves were plotted for one hour incubations using IL-8, TNF, IL-1, and ZAP as chemoattractants (Figures 7-9 and Table 7).

IL-8 produced the greatest response at all three concentrations of any of the three cytokines. The concentration dose of 100 ng of IL-8 attracted the maximal number of cells. This concentration was used for the following priming studies. The chemokinetic response to IL-8 was equivalent to the random migration results.

TNF also induced a directed migration in all three concentrations tested. Maximum response was at 1.0 ng. The chemokinetic migrations were slightly lower than random migration determinations.

IL-1 attracted similar numbers of cells in the directed migration wells as the TNF at equivalent cytokine concentrations. Maximum directed migration was seen at 1.0 ng. IL-1 elicited the largest chemokinetic migration compared to all other chemoattractants.

TABLE 6.

Mean Number of Cells in Direct Migration Well Using 100 ng ZAP as Chemoattractant

Date	CHJ09	CGF76	Date	CDF97	CCH41
4-16	79	84	4-18	67	75
4-19	83	83	4-22	66	75
4-23	79	82	5-8	56	66
5-9	59	62	5-14	57	64
5-13	63	73	5-16	62	70
5-15	69	80			
5-17	77	76			
MEAN	72.7	77.1		61.6	70.0
S.E.M.	±3.4	±2.9		±2.3	±2.3
C.V.	12.5%	10.1%		8.2%	7.2%

Figure 7. Granulocyte migration response to IL-8.

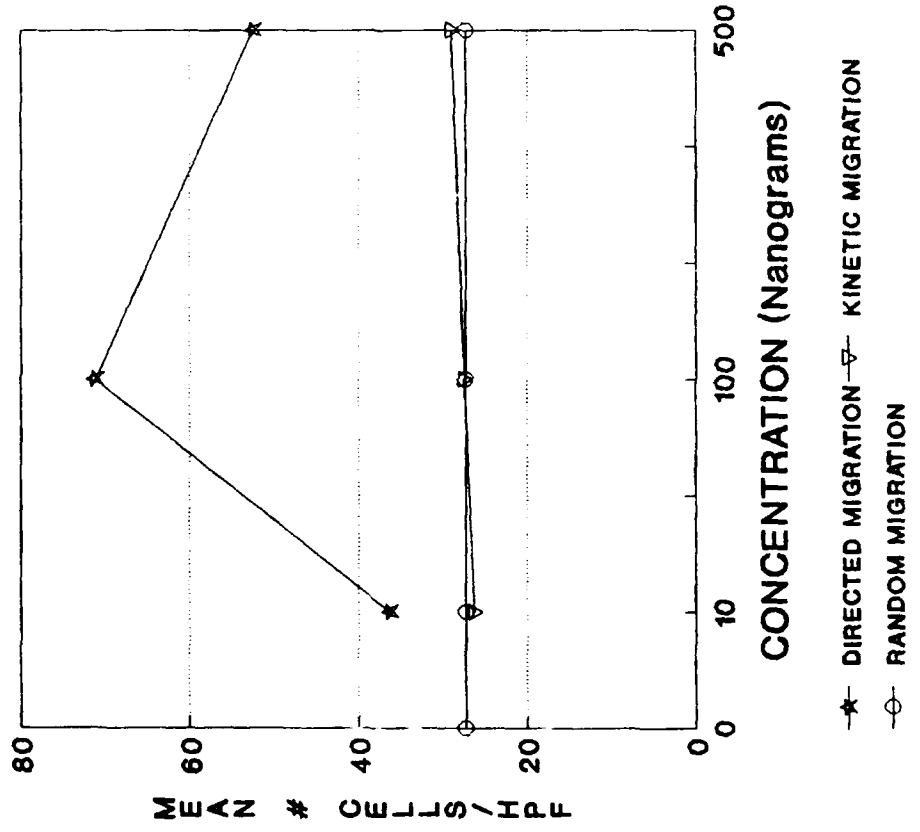

MEAN # CELLS/HPF
0
20
40
60
80
0
10
100
500
CONCENTRATION (Nanograms)
DIRECTED MIGRATION
KINETIC MIGRATION
RANDOM MIGRATION

Figure 8. Granulocyte migration response to TNF.

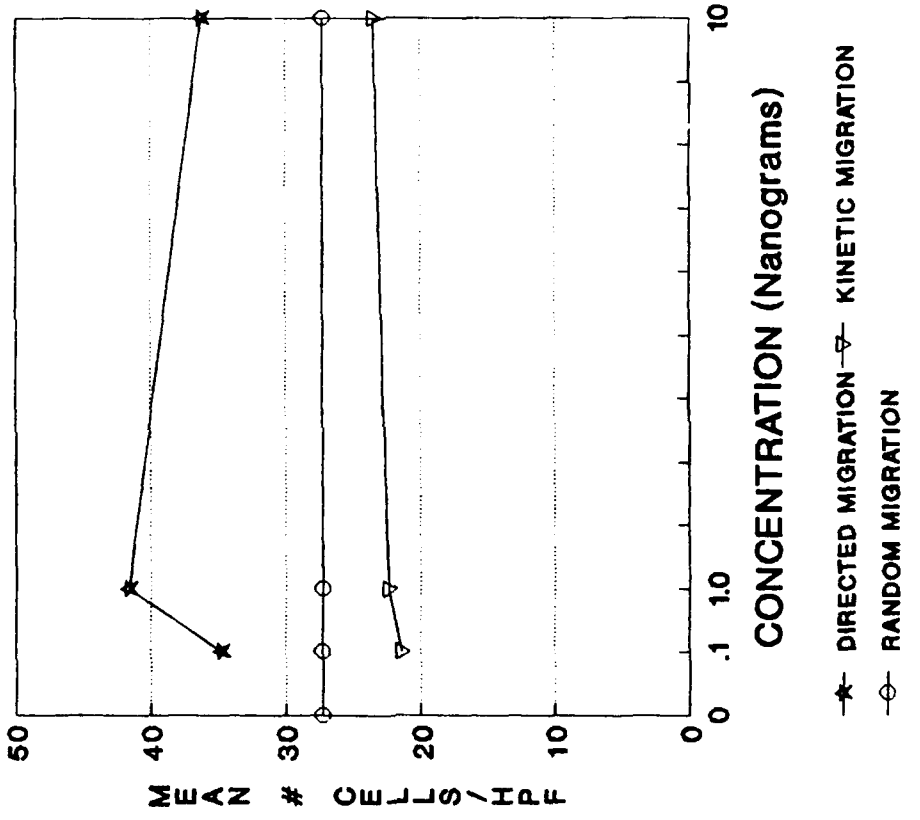

MEAN # CELLS/HPF
50
40
30
20
10
0
0
.1
1.0
10
CONCENTRATION (Nanograms)
DIRECTED MIGRATION
KINETIC MIGRATION
RANDOM MIGRATION

Figure 9. Granulocyte migration response to IL-1.

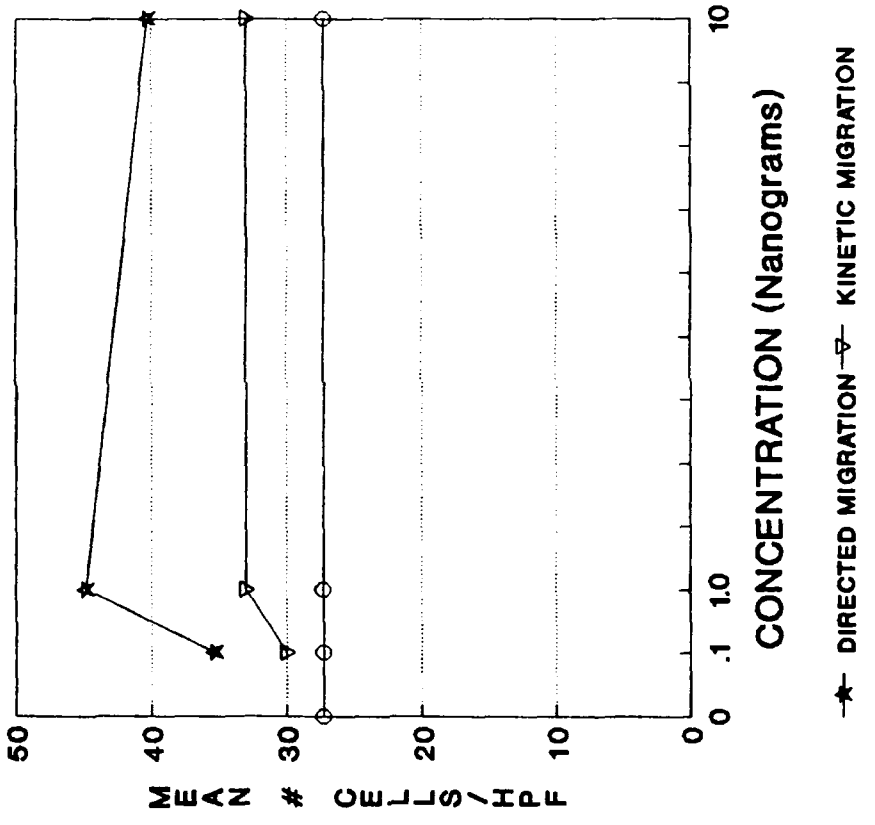

MEAN # CELLS/HPF
50
40
30
20
10
0
0
.1
1.0
10
CONCENTRATION (Nanograms)
DIRECTED MIGRATION
KINETIC MIGRATION
RANDOM MIGRATION

TABLE 7.

Mean Number of Cells in Random, Directed, and Chemokinetic Migration Wells Using Various Concentrations of IL-8, TNF, IL-1, and ZAP as Chemoattractants

	Random Migration	Directed Migration	Chemokinetic Migration
IL-8			
10 ng	27.3±0.9	36.2±3.3	26.2±0.6
100 ng	27.3±0.9	71.1±1.8	27.5±1.0
500 ng	27.3±0.9	52.5±3.3	29.0±1.4
TNF			
0.1 ng	27.3±0.9	34.8±4.1	21.4±2.0
1.0 ng	27.3±0.9	41.6±4.0	22.4±2.2
10 ng	27.3±0.9	36.2±3.7	23.5±2.0
IL-1			
0.1 ng	27.3±0.9	35.3±1.7	30.0±1.8
1.0 ng	27.3±0.9	44.8±1.6	33.0±2.8
10 ng	27.3±0.9	40.3±1.7	33.0±2.7
ZAP	27.3±0.9	56.6±4.7	36.4±3.1

Priming Studies

The granulocytes were primed with TNF and IL-1 for 30 minutes at 37°C with 0.1, 1.0, and 10 ng concentrations. Each specimen was loaded into the chemotaxis chamber and incubated an additional hour using 100 ng of IL-8 as the chemoattractant (Figures 10-11 and Table 8).

TNF primed cells were attracted in greater numbers than IL-1 primed cells at similar concentrations of cytokine. TNF primed cells showed a maximum response at 1.0 ng, only slightly higher than the response at 0.1 ng. The responses at 0.1 and 1.0 ng of TNF were significantly greater than the 37°C primed control ($p=0.0026$ and $p=0.0085$, respectively). All three values were higher than the direct TNF chemotaxis results.

Sufficient data points were not generated using IL-1 to warrant statistical comparison. However, 0.1 ng IL-1 primed cells showed a slight increase over the 37°C control. Cells primed with 1.0 and 10 ng of IL-1 exhibited less directed migration than the 37°C primed control. The chemokinetic migration at all three concentrations was similar to random migration. All three concentration responses were lower than the responses by the direct IL-1 chemotaxis assay. Cells that were primed with 0.1, 1.0, and 10 ng of IL-1 and then stimulated with 100 ng of IL-8

Figure 10. Granulocyte migration response to TNF priming and 100 ng IL-8 as chemoattractant.

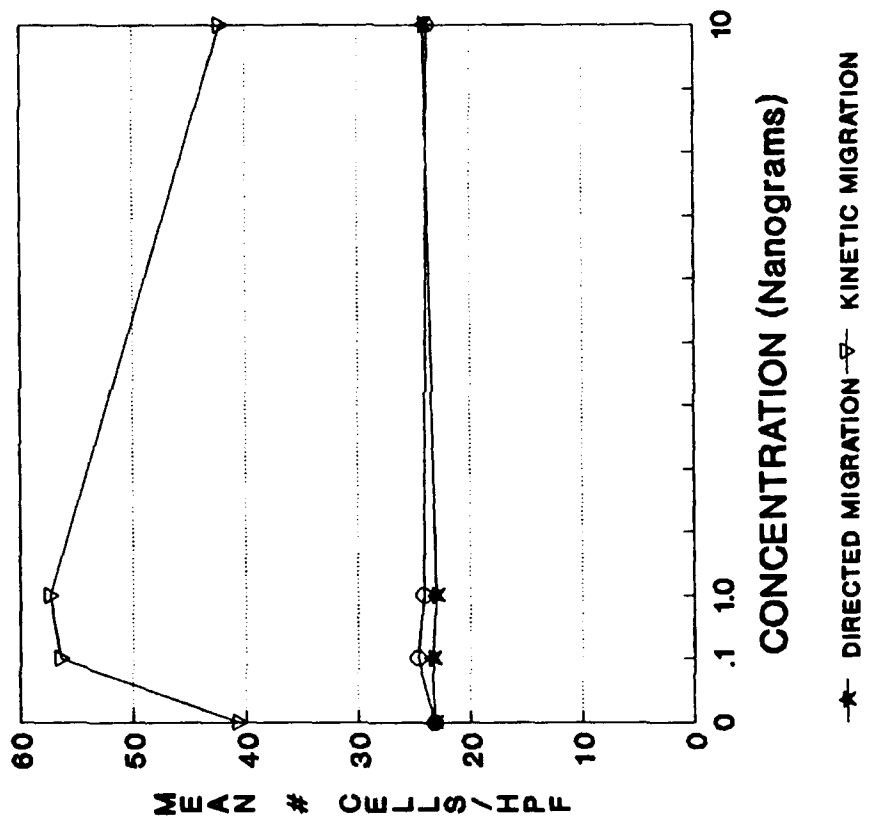

MEAN # CELLS/HPF
60
50
40
30
20
10
0
0
.1
1.0
10
CONCENTRATION (Nanograms)
DIRECTED MIGRATION
KINETIC MIGRATION
RANDOM MIGRATION

Figure 11. Granulocyte migration response to IL-1 priming and 100 ng IL-8 as chemoattractant.

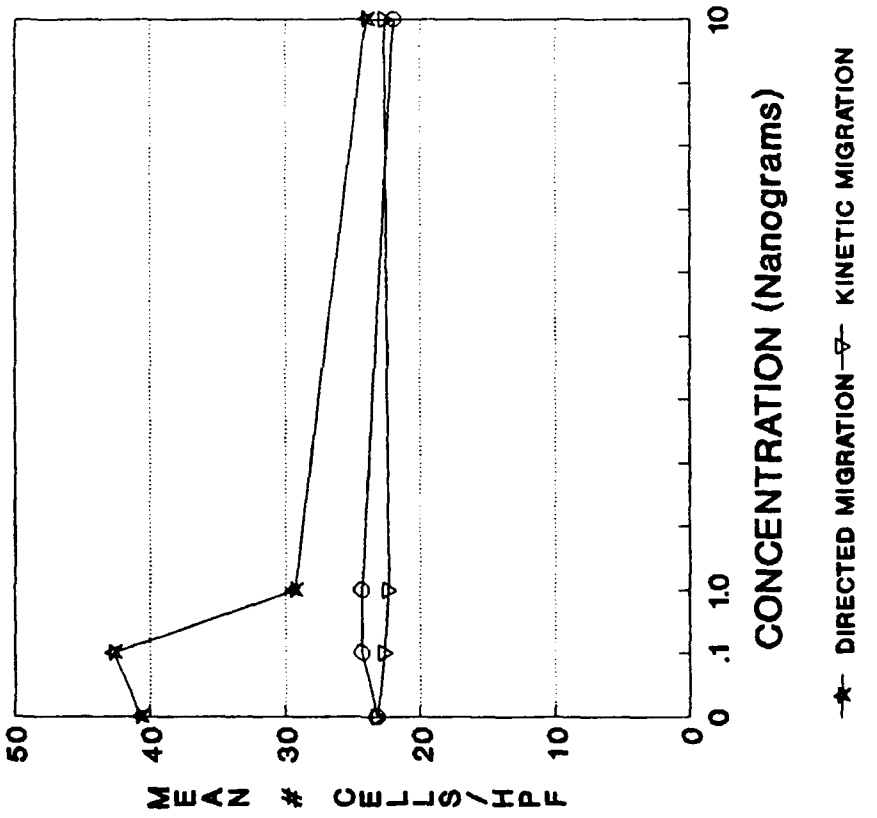

MEAN # CELLS/HPF
50
40
30
20
10
0
0
.1
1.0
10
CONCENTRATION (Nanograms)
DIRECTED MIGRATION
KINETIC MIGRATION
RANDOM MIGRATION

TABLE 8.

Mean Number of Cells in Random, Directed, and Chemokinetic Migration Wells Using Various Concentrations of TNF and IL-1 Priming for 30 Minutes, and 100 ng IL-8 as Chemoattractant

	Random Migration	Directed Migration	Chemokinetic Migration
TNF			
37C	23.2±1.1	40.7±2.8	23.2±1.0
0.1 ng	23.3±1.5	56.4±3.4	24.7±1.6
1.0 ng	23.0±1.9	57.3±4.6	24.1±2.0
10 ng	24.1±1.5	42.2±5.3	23.8±1.9
IL-1			
37C	23.2±1.1	40.7±2.8	23.2±1.0
0.1 ng	24.3±1.2	42.7±2.4	22.7±1.3
1.0 ng	24.3±1.3	29.3±3.0	22.3±0.7
10 ng	24.0±1.0	28.7±2.4	22.7±1.2

had lower directed migration counts than when 100 ng of IL-8 was used as an unprimed chemoattractant.

DCF Hydrogen Peroxide Production

Direct Stimulation Studies

Variability of the Method

The coefficient of variation and standard error of the mean for replicate determinations from the four animals were calculated. Each specimen was stimulated with 100 ng of PMA and measured after a fifteen minute incubation at 37°C (Table 9).

Dose Response Curves

Dose response curves were plotted for IL-8, TNF, and PMA induced hydrogen peroxide production at various time intervals (Figures 12-13 and Table 10).

At 10 ng concentrations IL-8 and TNF stimulated equivalent hydrogen peroxide production at each time interval. At 100 ng, IL-8 induced a significantly greater response than TNF at 15, 30, and 45 minutes ($p=.0028$, .0055, and .023 respectively).

The maximal response by IL-8 was at 500 ng at 45 minutes. This response was higher than any of the TNF

TABLE 9.

Periodic Measurements of Per Cent Change in Fluorescence of Four Canine Cell Suspensions Stimulated by 100 ng PMA

Animal	CHJ09	CGF76	CCH41	CDF97
Date:				
3-20	NT	NT	11.8	9.6
3-21	18.7	17.4	NT	NT
3-22	NT	NT	NT	9.0
3-25	17.6	16.0	11.2	7.8
3-26	22.6	26.0	14.5	8.0
3-27	20.2	22.5	NT	NT
3-28	NT	NT	9.5	10.2
4-01	16.9	15.6	13.2	12.6
4-03	19.9	20.1	10.5	NT
4-05	20.5	25.6	15.0	9.5
4-09	20.1	23.6	12.9	11.8
4-11	20.4	22.1	NT	NT
4-16	20.2	22.0	NT	NT
MEAN	19.7	21.1	12.3	9.8
S.E.M.	±0.5	±1.2	±0.7	±0.6
C.V.	8.1%	17.6%	15.5%	17.3%

NT = not tested

Figure 12. IL-8 stimulated hydrogen peroxide production dose response curve.

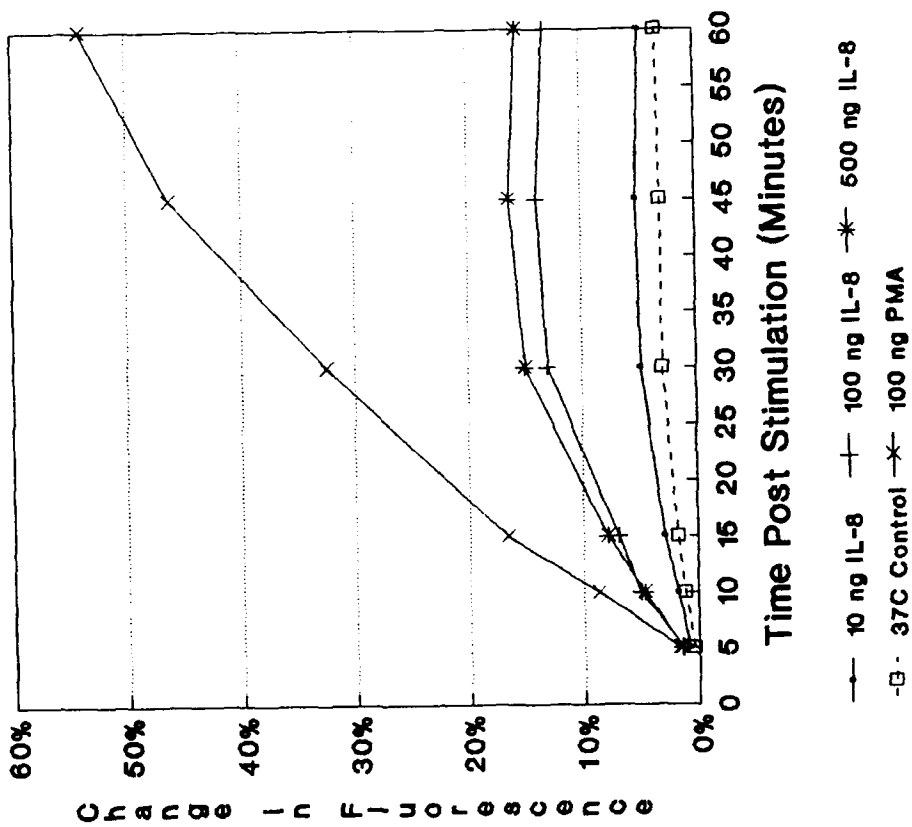

Change in Fluorescence
60%
50%
40%
30%
20%
10%
0%
0 5 10 15 20 25 30 35 40 45 50 55 60
Time Post Stimulation (Minutes)
10 ng IL-8
100 ng IL-8
600 ng IL-8
37C Control
100 ng PMA

Figure 13. TNF stimulated hydrogen peroxide production dose response curve.

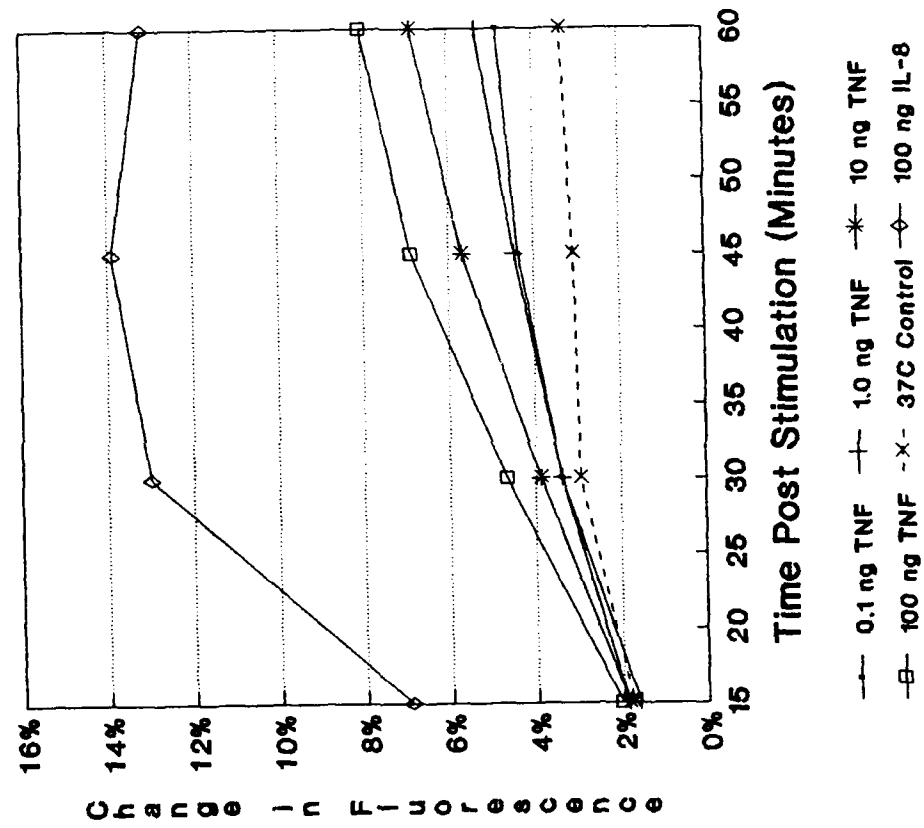

Change in Fluorescence
16%
14%
12%
10%
8%
6%
4%
2%
0%
15
20
25
30
35
40
45
50
55
60
Time Post Stimulation (Minutes)
0.1 ng TNF
1.0 ng TNF
10 ng TNF
100 ng TNF
37C Control
100 ng IL-8

TABLE 10.

Mean Per Cent Change in Fluorescence of Four Canine Cell Suspensions Stimulated by Various Concentrations of IL-8, TNF, and PMA Measured at Several Time Intervals Post Stimulation

Time (minutes)	5	10	15	30	45	60
IL-8						
10 ng	0.8 ±0.1	1.8 ±0.2	2.9 ±0.3	4.8 ±1.0	5.2 ±1.3	4.8 ±1.1
100 ng	1.4 ±0.2	5.0 ±0.6	7.0 ±1.0	13.0 ±2.0	13.9 ±2.2	13.2 ±2.2
500 ng	1.5 ±0.2	4.7 ±0.7	7.9 ±1.2	15.0 ±2.0	16.3 ±2.1	15.6 ±2.2
TNF						
0.1 ng	NT	NT	1.9 ±0.2	3.4 ±0.5	4.4 ±0.9	4.9 ±1.0
1.0 ng	NT	NT	1.6 ±0.2	3.4 ±0.7	4.5 ±1.2	5.4 ±1.5
10 ng	NT	NT	1.9 ±0.1	3.9 ±0.5	5.7 ±1.0	6.9 ±1.6
100 ng	NT	NT	2.0 ±3.1	4.7 ±0.7	6.9 ±1.3	8.1 ±1.8
37C Control	0.4 ±0.0	1.2 ±0.1	1.8 ±0.2	3.0 ±0.4	3.1 ±0.5	3.4 ±0.5
100 ng PMA	1.7 ±0.1	8.6 ±0.9	16.6 ±0.8	32.5 ±2.1	46.3 ±3.6	54.0 ±5.1

NT = not tested

responses. The 100 and 500 ng IL-8 responses were significantly greater than the time-matched 37°C controls. P values for the 100 ng response at 15, 30, 45, and 60 minute intervals were .0021, .0025, .0030, and .0045, respectively. P values for the same intervals with 500 ng of IL-8 were .036, .029, .025, and .031, respectively. Although the response was slightly higher with 500 ng of IL-8, it was not significantly higher than the response with 100 ng. Therefore, 100 ng of IL-8 was chosen for stimulation in the following priming study.

The maximal response for TNF was at 100 ng at 60 minutes. The 30, 45, and 60 minute intervals using 100 ng of TNF were the only data points significantly increased ($p=.049$, .028, and .044) compared to the time matched 37°C controls. The 0.1 ng TNF concentration showed slightly decreased levels of production, although not significant.

PMA induced a greatly increased response compared to either cytokine. H2O2 production did not plateau and continued to increase through the 60 minute time interval. Data points at the 15 minute interval and higher were significant ($p<.0001$) compared to the 37°C controls.

Priming Studies

The cells were primed with 10 ng of TNF for 30 minutes

at 37°C and then stimulated with 100 ng of IL-8. For comparison, the cells were also primed with 10 ng TNF and then stimulated with 100 ng of PMA. The per cent change in H2O2 production was measured at fifteen minutes (Figure 14 and Table 11).

PMA produced greater responses than IL-8 in all three cases. TNF primed cells stimulated with either PMA or IL-8 produced results that were lower than the 37°C primed controls, although not significantly. Additionally, TNF primed cells demonstrated lower responses than direct stimulation with IL-8 and PMA. They were, however, higher than the unprimed, unstimulated 37°C control (p=.0024 and .045).

Figure 14. TNF primed and PMA/IL-8 stimulated hydrogen peroxide production.

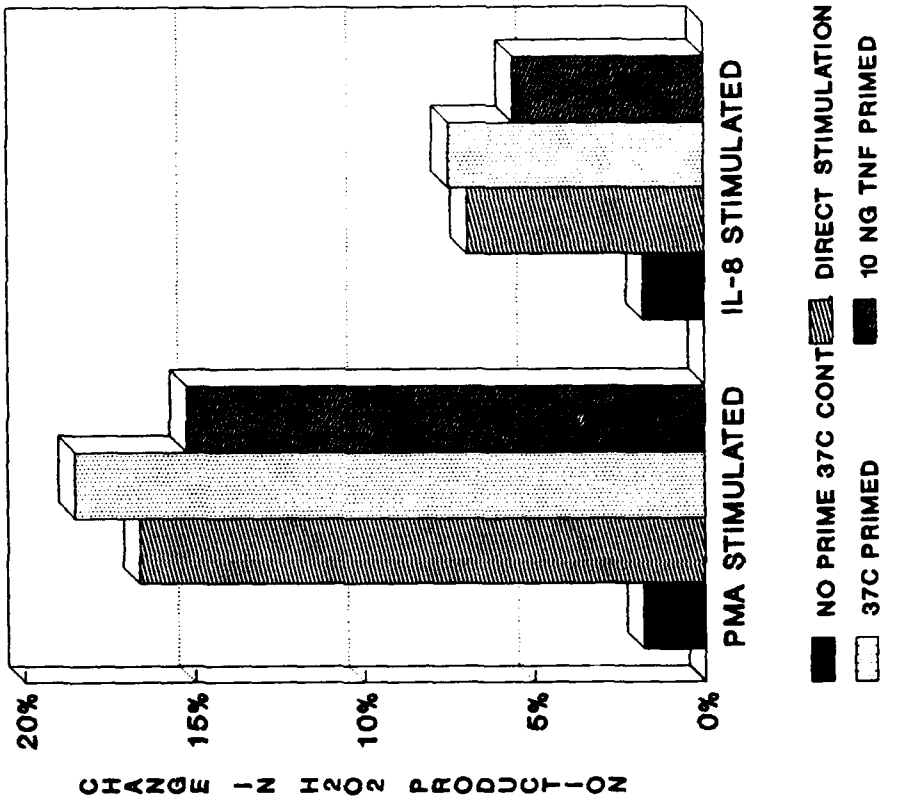

20%
15%
10%
5%
0%
CHANGE IN H_2O_2 PRODUCTION
PMA STIMULATED
IL-8 STIMULATED
NO PRIME 37C CONT
DIRECT STIMULATION
37C PRIMED
10 NG TNF PRIMED

TABLE 11.

Mean Per Cent Change in Fluorescence of Four Canine Cell Suspensions Unprimed and Primed with 15 Minutes of 37°C or 10 ng TNF, Followed by Stimulation with IL-8 and PMA

Primer:	No Prime	37°C Prime	10 ng TNF
Stimulant:			
37C Control	1.8±0.3		
100 ng IL-8	7.0±1.0	7.5±1.4	5.6±1.1
100 ng PMA	16.6±1.8	18.5±2.1	15.2±1.4

CHAPTER IV.

DISCUSSION

Conclusions

Vitamin B12 Binding Protein Release Assay

1. IL-1 and TNF primed cells did not elicit additional release of vitamin B12 binding proteins when compared to 37°C primed, IL-8 stimulated cells.
2. IL-1, TNF, IL-8, and PMA each stimulated significant release when used as direct stimulants in ten minute incubations compared to unstimulated controls.
3. The response of IL-1 and TNF primed neutrophils followed by stimulation with IL-8 was greater than the response seen with direct stimulation by either IL-1, TNF, or IL-8.

Chemotaxis Assay

1. At specific concentrations TNF and IL-1 primed cells were attracted in greater numbers than 37°C primed cells where IL-8 was used as the chemoattractant.
2. IL-1, TNF, IL-8, and ZAP prompted higher directed migration responses than random or chemokinetic migration responses.

3. Directed migration responses for each of the three cytokines and ZAP reached a plateau at higher concentrations.
4. Unprimed TNF and IL-1 directed migration responses peaked at lower concentrations than IL-8.

H2O2 Production Assay

1. Priming by TNF does not augment the neutrophil's ability to produce hydrogen peroxide when stimulated with PMA or IL-8.
2. IL-8, TNF, and PMA each stimulated increased H2O2 production, when used as direct stimulants, compared to unstimulated controls.
3. PMA produced the greatest response, followed by IL-8, and TNF.

Discussion

Priming neutrophils with IL-1 and TNF followed by IL-8 stimulation resulted in only limited increased cell functional capabilities. The cells' ability to migrate to IL-8 following TNF priming was enhanced compared to a 37°C primed, IL-8 stimulated control. IL-1 priming did not similarly enhance migratory response. Priming with IL-1 and TNF also did not induce additional degranulation when

compared to 37°C primed, IL-8 stimulated controls. Hydrogen peroxide production was not augmented by TNF priming.

Although the hypothesis was only partially supported by the data in this study, the premise of using cytokines in combination remains valid. IL-1 and TNF do not appear to play major roles in preparing the cell for synergistic response to IL-8 stimulation. Higher concentrations of IL-1 and TNF might have yielded the increased functional abilities sought, though higher concentrations would not be representative of the *in vivo* situation.

There remain many other cytokine combinations to be tested. A myriad of combinations must be required to properly attract and activate cells at just the right moment. Each cell function must be triggered and suppressed at the appropriate times to assure effective response to inflammation.

The data in this study indicated increased functional capability in all three assays when cells were directly stimulated by IL-8. The literature contains contradictory conclusions about the effect of IL-8, especially in the degranulation function (19). One of the possible reasons for the disparate results is the type of IL-8 used. Recombinant human IL-8 was used in this study. Other

studies have used "partially purified" (13), monocyte-derived (18), and endothelial-derived (15,16) forms of IL-8. Each type of IL-8 might bind with differing affinity to the IL-8 receptor.

A second possible explanation is the animal model used. This study used recombinant human IL-8 on canine neutrophils. Although IL-8 is not species specific (9), a different affinity might have resulted when the "cross species" IL-8 form was used.

Variability of the Methods

The variability of the three methods were high. The animals were monitored closely to preclude any infectious process that might activate the cells *in vivo*. Day to day variability was negated by using paired t-tests comparing in-run controls to each cytokine stimulated assay. The dog to dog variability was nullified by averaging the results of four animals, each run on at least two separate days. The variability was therefore most likely technique related. Every attempt was made to maintain the neutrophils in a quiescent state during the procedure. However, cell preparation and the assay require several centrifugations, mixing by vortex, and other manipulations that theoretically could activate the cells.

There is only one reference in the literature

regarding variability of this method. Gallin (27) reports a mean and S.E.M. of 64±21 for similarly stimulated human neutrophils. However, his study included only four data points.

Suggested Prospective Studies

The key to understanding neutrophil response to cytokine induced receptor transduction appears to lie in receptor quantity and affinity for the agonist.

Since priming the cells did not result in enhanced function when stimulated with IL-8, it is possible that the cell down-regulated IL-8 receptors in response to the priming cytokine. It is not clear whether the cell upregulated primer receptors to the exclusion of IL-8 receptors, or whether all cytokine receptors, including primer, were downregulated. Receptor binding assays to quantitate individual cytokine receptors as well as total cell cytokine receptors would be helpful. Binding of the first agonist could also alter the affinity for subsequent cytokines. An assay to determine the receptor affinity and any resultant change would also be enlightening.

Additionally, each cytokine binds different receptors but share a common protein in the next step of the pathway; protein kinase C. It would be helpful to know if this protein has been maximized within a quantitative range by

the first cytokine and is incapable of additional stimulation of similar magnitude by further cytokines.

Receptor studies might also help determine the up- or down-regulation of cell receptors in response to the changes in cytokine concentration. As the cell moves toward higher cytokine concentration it could be upregulating the receptors to magnify the migratory response. Beyond the optimal concentration, the cell might downregulate receptors. The cell's migratory response is probably a complex interaction of three mechanisms involving cytokine concentration, receptor affinity and quantity.

Determining the physiologic levels of each of the cytokines at the inflammatory site and at several distal points could clarify the interplay of sequentially acting cytokines. Although this study used pharmacologic levels of cytokine to mimic the action of physiologic levels, the levels at various sites should be proportional.

Finally, a study to determine the effect of cell warming should be undertaken. It is possible that receptors are being internalized as a result of cell warming and diminishing the effect of cytokine stimulation. The 37°C priming or warming of the cells produced an unexpected increase in hydrogen peroxide production. This study was conducted using only a

30 minute priming time. To fully understand the priming effect alone, a study using 37°C primed cells over a full course of priming times should be conducted. Such data could supplement the data in this study to determine the contributing influences of 37°C priming, TNF priming, and IL-8 stimulation on the cell's production of hydrogen peroxide.

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